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Combined demographic, ecological and genetic tools reveal connectivity within a fragmented butterfly population

Irene Piccini [a,b,c], Irene Pellegrino [d,*], Davide Bellone [e], Marco Cucco [d], Viviana Di Pietro [f], Flavio Mignone [g], Martina Nasuelli [d], Simona Bonelli [a]

[a] Department of Life Sciences and System Biology (DBIOS), University of Turin, Turin, Italy
[b] Department of Zoology, Poznań University of Life Sciences, Poznań, Poland
[c] Department of Science and Technological Innovation (DiSIT), University of Piemonte Orientale, Alessandria, Italy
[d] Department for Sustainable Development and Ecological Transition (DiSSTE), University of Piemonte Orientale, Vercelli, Italy
[e] Agroécologie, INRAE, Institut Agro, Univ. Bourgogne, Univ. Bourgogne-Franche-Comté, Dijon 21000, France
[f] Department of Biology, KU Leuven, Leuven, Belgium
[g] SmartSeq srl., Alessandria, Italy

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ABSTRACT

Protected species require targeted studies to establish population sizes, their dispersal capacity and the possible genetic bottleneck effect that could impair the future of the remaining populations. In this study, we used the genotyping-by-sequencing technique (SNP: single nucleotide polymorphism) combined with Capture-Mark-Recapture (CMR) method to measure the dispersal ability and subpopulation sizes of the protected butterfly *Zerynthia polyxena* in 14 sites of NW Italian Alps. The study species form a metapopulation divided into several small subpopulations (ranging from 90 to 859 estimated subpopulation sizes). The species showed low mobility, likely due to habitat fragmentation, with individuals travelling an average of 80 m from their original capture site. SNPs highlighted high observed heterozygosity, low genetic substructuring at short distances, and large genetic differentiation only for individuals found in sites located at 46 km distance, suggesting high gene flow at short distances and limited gene flow between distant populations. Although CMR data did not detect movement beyond 700 m, the genetic results revealed remarkable connectivity among nearby subpopulations. The results indicate that gene flow still exists among the subpopulations despite the fragmented habitat. Considering that the species is locally threatened by habitat loss due to the planning of a European high-speed railway, our data suggest that it is not too late to adopt conservation measures. In conclusion, combining genetic and ecological studies can be utilised to design proper conservation plans for protected and threatened insect metapopulations.

1. Introduction

The evaluation of the conservation status of protected species requires well-focused investigations to establish their population stability (Henry and Anderson, 2016), dispersal capacity (Hanski et al., 2000) as well as the existence of genetic bottlenecks that could

* Correspondence to: Department for Sustainable Development and Ecological Transition (DiSSTE), University of Piemonte Orientale, Piazza Sant'Eusebio 5, Vercelli 13100, Italy.
E-mail address: irene.pellegrino@uniupo.it (I. Pellegrino).

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impair the persistence of remaining populations (Brookes et al., 1997). These investigations are crucial when conservation efforts are directed towards protected species characterised by small populations, often at high risk of extinction (Hanski, 1998; Pimm et al., 1988; but see: Shahabuddin and Ponte, 2005). Moreover, habitat degradation and/or fragmentation often exacerbated the local extinction risk of species (Hanski, 1999; Komonen et al., 2008). In such habitats, species features such as limited mobility can lead to a drastic decrease in their survival probability, as suggested by population dynamics models (Barbaro & van Halder, 2009; Thomas, 2000). Indeed, species with low mobility are more subject to population decline compared to high-mobility ones, which can more easily move and colonise new portions of habitat (Magura et al., 2015). Characterising the population dynamics, mobility and the genetic diversity of vulnerable species populations is crucial to determine potential extinction risks.

Large habitat patches, rich in resources and breeding sites, have been traditionally considered able to support high biodiversity (Wilson and Willis, 1975). More recent research has shown that also a mosaic of heterogeneous small patches can support higher biodiversity in comparison with large and homogeneous patches (e.g. Riva and Fahrig, 2023). However, habitat fragmentation can lead to detrimental effects when connectivity between patches is disrupted, which in species with low mobility might have negative genetic consequences. Indeed, a lack of connectivity for low-mobility species can limit or interrupt gene flow among small populations (Trense et al., 2022). Moreover, the size reduction of isolated populations that reside in small, fragmented patches could cause inbreeding depression and a reduced capacity of the populations to adapt to environmental changes (Frankham et al., 2017; Nieminen et al., 2001). The level of reduction of gene flow depends on several factors, including the number and distribution of population fragments, the distance between populations, the dispersal ability of the species and the type of barriers among sites (Frankham et al., 2017; Habel et al., 2010).

Insect communities rely on temporal and spatial heterogeneity and habitat connectivity for their long-term survival (Samways et al., 2020). Among insects, butterflies are often the most studied group, and their protection can help maintain vital and functional ecosystems for other species (Launer and Murphy, 1994; New, 1997). Butterflies are known for playing a pivotal role as pollinators within ecosystems (Macgregor et al., 2015), and are considered indicator species of the quality of the habitat in which they reside (Maes and Van Dyck, 2001; Thomas, 2005). Determining butterfly population size and mobility is difficult and work-demanding but it is essential to understand their population conservation status and to identify present and future threats (Bonelli et al., 2013; Casacci et al., 2015; Wahlberg et al., 2002). Among different threats, the low levels of heterozygosity shown by butterfly populations with few individuals and scarce mobility can increase the risk of local extinction (Nieminen et al., 2001; Saccheri et al., 1998). Low genetic variation can occur in highly fragmented habitats by interrupting gene flow, especially in butterfly populations with low mobility (Dapporto, 2010; Schlaepfer et al., 2018) cascading in the formation of smaller independent populations, each facing a greater risk of local extinction (Roland and Matter, 2007). When the number of remaining individuals in the distributional range of butterfly species decreases (Warren et al., 2021), or when the mobility is low (Trense et al., 2022) and the abundance of surviving subpopulations in a metapopulation system is small (Schulz et al., 2020), conservation interventions to reverse this scenario are required. Successful management programs must include data on the genetic structure and heterozygosity of the butterfly populations to maximise the balance between increasing genetic diversity, to avoid inbreeding depression, and prevent outcrossing depression and genetic disturbance (Bohling, 2016; Davis et al., 2021; Liddell et al., 2021; Ralls et al., 2018, Sucháčková Bartoňová et al., 2023). In addition, to ensure the long-term vitality of a population, it is essential to evaluate the ability to colonise new sites and to connect with residing individuals of the same species. Several direct and indirect methods have been employed to estimate dispersal ability such as Capture-Mark-Recapture (hereafter CMR) used to identify movements that potentially drive gene flow, and molecular estimates of gene flow among populations (Casacci et al., 2015; Stevens et al., 2010).

This study reports data on *Zerynthia polyxena*, a protected butterfly (listed in Habitats Directive 92/43/EEC, Annex IV) constituting a metapopulation of several subpopulations in NW Italian Alps. The study areas are typical alpine valleys characterised by high anthropic disturbance. Such disturbance, along with agriculture abandonment, has led to widespread tree encroachment and habitat loss for open habitat species in some of these valleys (Bonelli et al., 2011). *Z. polyxena* is an ecotonal species, therefore conservation measures could include protection or expansion of clearings and ecotonal areas (Piccini et al., 2022a), and create new ecotonal vegetation corridors between currently occupied sites (Piccini et al., 2022b). A subpopulation of the species in Susa Valley is currently threatened by the construction site of a megaproject (Piccini et al., 2022a). Here, we use a multidisciplinary approach, using genome-wide SNP markers and CMR, to assess the genetic structure and the population dynamics of *Z. polyxena* in fragmented habitats at its altitudinal distribution limit, in NW Italy Alps. Considering the small site size and the large fragmentation that characterised the NW Alps due to agro-pastoral abandonment (Orlandi et al., 2016), we hypothesised that: 1) subpopulations consist of few individuals and show a low dispersal ability. Then, considering the expected small subpopulation sizes and low mobility, we hypothesised 2) a low heterozygosity of subpopulations living in separated clearing areas that might present possible past genetic bottlenecks, and 3) a low gene flow between current subpopulations. The results of our study will provide a scientific background for designing adequate measures and conservation plans to minimise the impact of potential threats (such as megaproject construction sites) on *Z. polyxena* butterfly subpopulations.

2. Methods

2.1. Study area

The study area was located in the north-western Italian Alps (WGS84: 45070469 N 6590147 E) with an altitude ranging from 600 to1285 m asl. The study area was divided into four areas: the core area of the species, where the CMR study was conducted (High and Middle Susa Valley), and three other areas where the species is present (Low Susa Valley, Sangone Valley and Lanzo Valley; Fig. 1;

Table 1). The area is dominated by hay meadows belonging to Festuco-Brometea and Molinio-Arrhenatheretea classes and abandoned chestnut forests originally cultivated for both fruit and timber production managed mainly with copping practice (Piccini et al., 2023).

2.2. Study species

Zerynthia polyxena is a univoltine butterfly (Bonelli et al., 2018; Čelik, 2012), belonging to the family Papilionidae. In Italy, the adults fly from March until the beginning of June and colonise a mosaic of small ecotonal sites (Piccini et al., 2022a). The females oviposit on *Aristolochia pallida* in the study area, and it is locally monophagous (Piccini et al., 2021), laying either a cluster of eggs or a single egg (Batáry et al., 2008). The species overwinters as pupae on the ground (Balletto et al., 2023). It is largely present in Europe, with fragmented distributional ranges covering the Southern and Central parts of the continent, also reaching the Balkans and Asia Minor (Fig. S1 in Supplementary material). In Italy, it is restricted to the north, the central and south populations have been recently described as a new endemic species *Zerynthia cassandra* (Zinetti et al., 2013). In NW Alps, we sampled most of the known subpopulations (and some unknown subpopulations before this study) belonging to a metapopulation (Hanski, 1998) in 2019 (see Fig. S1 in Supplementary material). It is considered Least Concern both at Italian and European level, but with population trends declining due to isolation (Bonelli et al., 2018; Swaay et al., 2010). In the NW Italian Alps, a subpopulation is threatened by the expansion of the construction site of the High-Speed railway Turin-Lyon.

2.3. Estimation of subpopulation sizes

To investigate the size and dispersal ability of the species, we focused on the closest subpopulations in NW of the Italian Alps. Thus, we selected a subsample of sites that were distanced by 1–10 km (Table 1). Then, in those sites we carried out the Capture-Mark-Recapture (CMR) method to estimate the subpopulation sizes of the species. Capture events were conducted only under suitable weather conditions (no rain) over 86 days between March 18th and June 12th, 2019 (total of 57 days of captures when weather conditions were favourable). Each butterfly was caught with an entomological net and marked individually with a unique combination

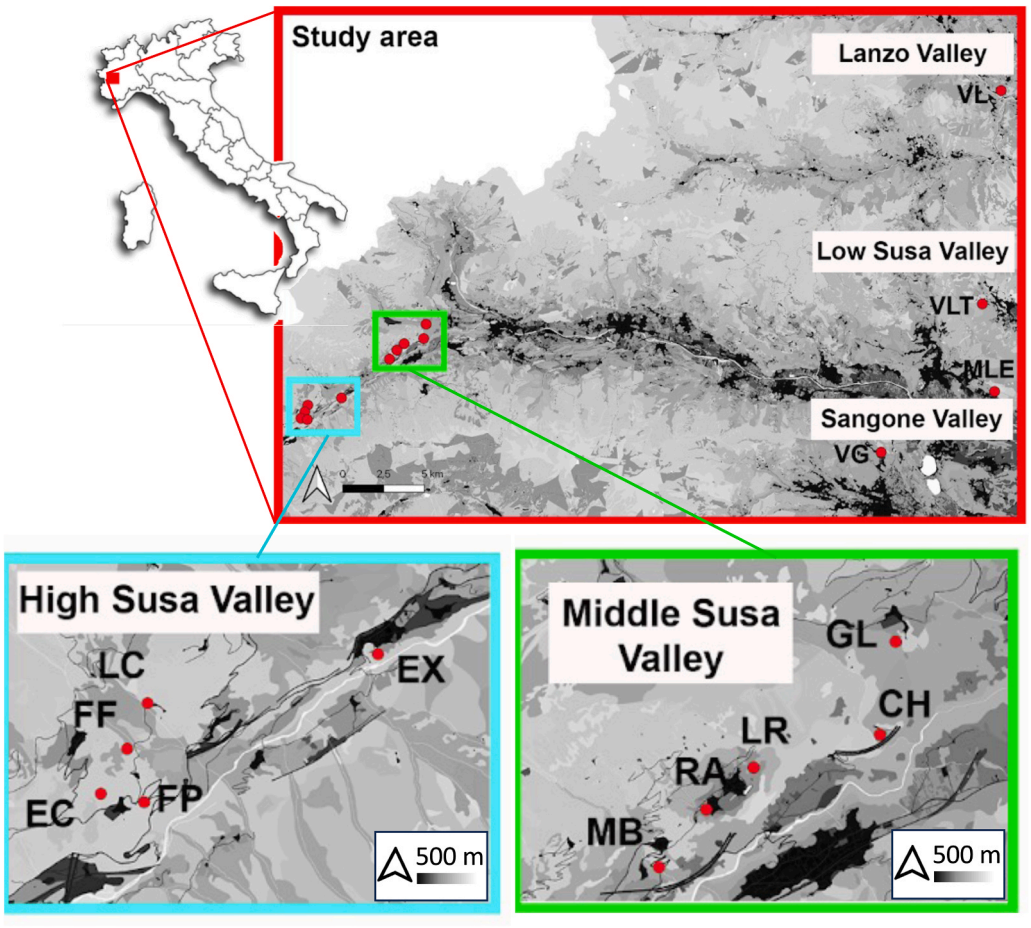

Fig. 1. Map of the study area. Sampling sites of *Zerynthia polyxena* are indicated with red dots. Green square encompasses Middle Susa Valley sites and the cyan one High Susa Valley sites (NW Italy).

Table 1
List of sampling sites in NW Italy with associated genetic variability in *Zerynthia polyxena* from genomic analyses and subpopulations studied for demographic and ecological investigation. For each site, the area, code, name and altitude are given, as well as genotyped individuals (N), expected (He) and observed (Ho) heterozygosity, allelic richness (Ar) and inbreeding coefficient (FIS). CMR indicates if a site was used in the Capture-Mark-Recapture study.

Areas	ID	Subpopulations/Sampling sites	Altitude (m a.s.l.)	N	H_E	H_O	Ar	F_{IS}	CMR
High Susa Valley	EC	Eclause	1250	13	0.254	0.3557	1.7787	-0.249	X
	EX	Exilles	860	9	0.244	0.3446	1.7631	-0.245	
	FF	Forte Fenil	1200	12	0.146	0.1968	1.5297	-0.155	X
	FP	Forte Fenil - Pramand	1150	8	0.294	0.4341	1.8478	-0.313	X
	LC	Le Combe	1230	9	0.285	0.4017	1.8617	-0.268	X
	Total			54					
Middle Susa Valley	CH	Chiomonte	650	11	0.228	0.3298	1.7380	-0.244	X
	GL	Giaglione	1050	13	0.231	0.3344	1.7316	-0.255	X
	LR	La Ramats	990	8	0.166	0.243	1.5360	-0.246	X
	SA	Sant'Antonio	1000	9	0.238	0.335	1.7632	-0.234	X
	MB	Maison-Baccon	890	10	0.267	0.3821	1.8108	-0.267	
	Total			51					
Low Susa Valley	ML	Milanere	400	9	0.207	0.2879	1.6625	-0.221	
	VLT	Val della Torre	1000	8	0.259	0.3803	1.7582	-0.299	
	Total			18					
Sangone Valley	VG	Valgioie	880	9	0.270	0.4023	1.7761	-0.319	
Lanzo Valley	VL	Val di Lanzo	630	8	0.218	0.3203	1.6468	-0.291	
		TOTAL		136					

of colours on the underside of the left hindwing using a non-toxic fine-tip permanent marker (Figure S2 in Supplementary material). For all individuals, we noted the identification code, sex, age (estimated using morphological conditions), behaviour before netting, GPS position (Garmin. eTrex 20 with a precision of 3 m) and the time (date, hour) of the capture/recapture event, following Parile et al. (2021) and Piccini et al. (2024). All individuals were immediately released at the same capture location.

POPAN, in the program MARK 8.0 (White and Burnham, 1999), was used to estimate the size of *Z. polyxena* subpopulations. We obtained the survival probability (φ), recruitment rate (probability of entrance: pent), capture probability (p) and the estimated subpopulation sizes (N). These parameters may be constant (.), dependent on sex (g) or on time (t). We identified the model with the lowest Akaike's Information Criterion (AIC) as the best-fitting model. The POPAN approach (Schwarz and Arnason, 1996) has already been employed for butterflies, whose population size changes daily mainly due to recruitment and deaths of adults (e.g. Čelik, 2012; Piccini et al., 2024). For certain sites (CH, LR+SA and LC), the available CMR datasets lacked the precision necessary to derive accurate estimates using the chosen model. Consequently, we calculated the total population size by multiplying the count of captured female butterflies in the site by a conversion coefficient (Mennechez et al., 2004). This coefficient was derived from sites where the local population size was determined using POPAN, and it represents the estimated number of individuals divided by the number of marked individuals.

The average life span of butterflies emerging discontinuously from pupae is calculated as $(1/(1-\varphi))-0.5$ (Nowicki et al., 2005). The average daily survival (φ) is a weighted mean of the POPAN daily values, weighted by the number of captures on a given day (Čelik, 2012).

2.4. Mobility

To evaluate the mobility of *Z. polyxena* adults, we used GPS data collected during CMR. We calculated the distance between consecutive captures on different days (D) as a straight line connecting two consecutive points of butterfly capture in QGIS 3.4 (QGIS Development Team, 2018). According to Jugovic et al. (2017), we calculated the mean, median and maximum distances separately for males and females. We modelled distances (D) in a generalised linear model (GLM, gamma, maximum likelihood fit), using sex as explanatory variable.

To understand the dispersal ability of females and males (Pennekamp et al., 2014), we estimated the probabilities of movements with respect to distance beyond those covered during the study. Thus, we calculated the negative-exponential function (NEF), according to Hill et al. (1996). All distances were grouped into 50 m classes, separately for each sex. The probability (P) of an individual moving a certain distance (D), beyond that covered during the study, was calculated as: P_NEF = a e–kD. While D′ represents the average distance moved by individuals, it is calculated by $D' = 1/k$ (Hill et al., 1996). Considering that log-transformed formulas can be expressed as linear relationships [i.e. ln(PNEF) = ln(a) − kD], and following Čelik (2012), the a and k parameters were estimated by LMs in R. Thus, the natural logarithms of the inverse cumulative proportions (ICP) of individuals moving certain distances (lnP) were regressed on distances (metres). Therefore, we modelled natural logarithms of ICP into a LM (Gaussian, maximum likelihood fit) where sex (as a categorical variable), metres (as continuous variables), and their interaction (sex x distance) were used as additive explanatory variables. We tested for the normality of the residuals (Shapiro–Wilk test: $p = 0.493$). Plots were drawn with the *ggplot2* (Wickham, 2016) and *visreg* (Breheny and Burchett, 2017) packages in R version 4.3.1 (R Core Team, 2023).

2.5. Genetic and genomic analyses

2.5.1. Biological sample collections and DNA extraction

The biological tissues were collected from 140 individuals in 14 subpopulations (Table 1 and Table S1 in Supplementary material) by cutting the distal portion of one or two legs; this method does not compromise the vital function of the individual (Crawford et al., 2013; Koscinski et al., 2011). No individual was sacrificed for this research.

We modified the standard DNA extraction protocol of the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany) to increase the overall DNA yield. Briefly, we applied a long incubation for pre-lyse (at 56 °C overnight) and for lyse (70 °C for 2 h) phases. Two successive elutions (30 and 35 μL respectively) were performed with a heated elution buffer (70 °C) and an incubation step of 3 min followed by centrifugation (11,000*g* for 1 min).

2.5.2. Sanger sequencing

We analysed one mitochondrial and one nuclear marker to investigate the genetic diversity of subpopulations in Susa Valley and surroundings and compare the haplotypes with data already published in the literature for this species. Because these markers have a low intraspecific variability especially at small geographical scale (Hebert et al., 2004), we only sequenced a subset of individuals belonging to 12 subpopulations for the COI gene and for the nuclear *wingless* gene. A portion of 649 bp of Cytochrome c oxidase (COI) was amplified and sequenced using the primers LepF1 and LepR (Dincaă et al., 2011) in 43 samples (Table S1 Supplementary material) following the PCR and thermal protocols published in Pellegrino et al. (2017). The *wingless* (*wg)* gene was sequenced in 53 individuals (Table S1 in Supplementary material) with the primers wg_F29 and wg_R382 following the protocols published by Zinetti et al. (2013). Sequencing was performed by Macrogen Europe Laboratory in The Netherlands. Electropherograms were verified with FinchTV v1.5 (Geospiza, Seattle, WA, USA) and were aligned with published sequences (from Dapporto et al., 2019; Dincă et al., 2011, 2021; Zinetti et al., 2013) using Mega X (Kumar et al., 2018). Median-Joining haplotype networks (Bandelt et al., 1999) were calculated with PopArt software (http://popart.otago.ac.nz).

2.5.3. Genotyping-by-sequencing (GBS) library preparation and sequencing

We analysed 140 individuals from 14 different subpopulations (Table 1, Supplementary material S1, Fig. 1) following the protocol of Elshire et al. (2011) with some modifications developed by SmartSeq s.r.l. (Novara, Italy) and published in Pellegrino et al. (2016). Briefly, after estimation of the concentration of extracted DNA by fluorescence (Qubit 4, Thermo Fisher Scientific), 100–250 ng of DNA was digested with *PstI*-HD restriction enzyme (New England Biolabs), and two different types of adapters were ligated with T4 ligase (New England Biolabs). Samples were pooled in libraries and purified through NucleoMag NGS kit (Macherey-Nagel, Düren, Germany), each library was then amplified with adapters for the Illumina sequencing, according to the original protocol by Elshire et al. (2011), and finally products were size-selected, with a range between 200 and 400 bp, and pooled using the same magnetic beads kit

The concentration of samples was adjusted by dilution or evaporation (SpeedVac Thermo Fisher Scientific, Carlsbad, CA), and then samples with similar concentrations were grouped in the same index group.

The libraries were purified with NucleoMag NGS kit and, finally, sequenced in three runs with an Illumina MiSeq (150 bp single-end reads), getting about 25 million reads each run. SNPs identification was conducted using a custom bioinformatics pipeline by bioinformatic SmartSeq s.r.l. After demultiplexing the individuals, all reads with mismatches in the adapter or sticky end sequences were discarded. All reads were aligned to identify clusters representing the same genome region. One read for each cluster was selected as a reference; then clusters were compared to the reference to identify SNPs.

We considered only SNPs and indels meeting the following criteria: (1) a depth of at least 40 reads, (2) at least 4 reads with the variant, and (3) the allelic variant present on at least 2 samples. The SNP dataset obtained from the previous pipeline was further filtered using the software PLINK 1.07 (Purcell et al., 2007). SNPs with a minor allele frequency (-maf) as 0.05, maximum per-SNPs missing (-geno) as 0.01 and maximum per-person missing (-mind) as 0.4 were removed. Finally, the SNP set was also pruned for linkage disequilibrium (LD) by excluding markers in strong LD (pairwise genotype correlation r2 $>$ 0.5) in a window of 50 SNPs (sliding window overlap 5 SNPs at a time). Output files were converted to other formats for downstream analyses using PDGspider 2.0.7.2 (Lischer and Excoffier, 2012).

The selected SNPs were analysed with R version 4.3.1 implemented in RStudio version 2023.9.1.494 (Posit team, 2023), with packages *adegenet* (Jombart, 2008; Jombart and Ahmed, 2011), *genepop* (Rousset, 2008) and *hierfstat* (Goudet, 2005). In particular, we calculated observed heterozygosity (Ho), expected heterozygosity (genetic diversity) and F-statistics F_{IS}, F_{IT} and F_{ST} (Nagylaki, 1998; Wright, 1949) with *hierfstat*. Subpopulation structure was explored with *adegenet* by performing a Discriminant Analysis of Principal Components (DAPC; Jombart et al., 2010) on groups defined by sampling site.

Isolation by distance (Rousset, 1997) was tested between sampling sites using *genepop* package in R. Mantel tests were performed between matrices of linearized pairwise FST and geographical distances setting the number of repetitions to 1000.

The stochastic model of potential directions of gene flows between subpopulations based on the effective number of migrants per generation was implemented using the divMigrate (Sundqvist et al., 2016) function in the R package *diveRsity* (Keenan et al., 2013). To calculate directional relative migration, we utilised the Nm statistic (Alcala et al., 2014) that combines elements of G_{ST} and D.

Finally, we estimated the effective subpopulation size (*Ne*), defined as the size of an ideal panmictic subpopulation that experiences as much genetic drift as an actual subpopulation regardless of its census size (Hamilton, 2021), using the Linkage Disequilibrium Model (LDM) with a critical value of 0.02 in NeEstimator v2.1 (Do et al., 2014). We grouped into two areas, High and Middle Susa Valley (Table 1).

Table 2
Zerynthia polyxena marked individuals, percentage of recaptured individuals and within brackets number of recaptured individuals (N), percentage of total recapture events and within brackets total number of recapture events (N), estimated subpopulation sizes (N_{est}) ± standard deviation, separated by females (F), males (M) and total, density (N_{est}/ha) and estimated life span (days; * life span estimated using only data on males). Site abbreviations as given in Table 1.

Sites	Marked individuals			% Individual recaptured (N)			% Recaptures (N)			Estimated subpopulation sizes (N_{est})			Density	Life span
	F	M	TOT	F	M	TOT	F	M	TOT	F	M	TOT		
CH	31	26	57	41.9 (13)	30.8 (8)	36.8 (21)	61.3 (19)	53.9 (14)	57.9 (33)	57.77 ± 15.83	32.12 ± 5.37	89.89	24.68	3.74*
GL	34	32	66	32.4 (11)	31.3 (10)	37.9 (21)	52.9 (18)	43.8 (14)	48.5 (32)	51.08 ± 8.97	68.83 ± 12.18	119.91	11.29	3.20
LR+SA	180	213	393	12.8 (23)	22.1 (47)	20.2 (70)	12.8 (23)	26.3 (56)	20.1 (79)	335.44 ± 91.94	523.97 ± 64.55	859.41	34.04	4.55*
FF+FP	109	110	219	27.5 (30)	22.7 (25)	26.0 (55)	32.1 (35)	29.1 (32)	30.6 (67)	242.50 ± 30.15	235.12± 29.18	477.62	24.28	5.53
LC	16	20	36	0 (0)	40.0 (8)	27.8 (8)	0 (0)	40.0 (8)	22.2 (8)	29.82 ± 8.17	42.88 ± 0.19	72.70	60.33	-
TOT	**370**	**401**	**771**	**20.8 (77)**	**24.5 (98)**	**25.0 (175)**	**25.7 (95)**	**30.9 (124)**	**28.4 (219)**	**716.61**	**902.92**	**1619.53**	**30.92**	**4.26**

3. Results

3.1. Estimation of subpopulation sizes

In total, 771 individuals were marked (CH: 57, GL: 66, LR+SA: 393, FF+FP: 219 and LC: 36) and 175 recaptured individuals with a total of 219 recapture events, with some individuals recaptured more than one time (Table 2). Out of 771 captured individuals, 371 were females and 400 males, while out of 193 recaptured individuals (25 %), 83 were females (22.4 %) and 110 males (27.5 %; Table 2). The sex ratio was evaluated as 1:1.1 in favour of the males.

Each subpopulation has a different best model through which we estimated survival probability (φ), capture probability (p) and recruitment rate (pent; Table S2 in Supplementary material). For CH and LR+SA female subpopulations we used the conversion coefficient calculated for the other subpopulations, while for LC subpopulation we used it to estimate both females and males (Table S3 in Supplementary material). The total population size for all the subpopulations combined was 1619.53 individuals, divided into CH 89.89, GL 119.91, LR+SA 859.41, FF+FP 477.62 and LC 72.70 individuals (Table 2). The estimated average lifespan was 4.26 days (CH males: 3.74, GL: 3.20, LR+SA males: 4.55, FF+FP: 5.53; Table 2). The minimal convex hull for each capture and recapture event in each subpopulation was calculated (Table S2 in Supplementary material), resulting in an estimated butterfly density of about 31 individuals/ha (min 11 in GL; max 60 in LC; Table 2), and 13 captured individuals/ha (min 6 in GL; max 30 in LC).

3.2. Mobility

For the recaptured individuals we recorded 264 movements, 144 for females and 120 for males. Median, mean and max travelled distances were 51.7 m, 79.8 m, and 628 m, respectively. For males, the values were 45.1 m, 67.7 m and 396 m, respectively, while for females, the values were 55.9 m, 89.9 m and 628 m, respectively. Mobility varied in relation to sex, where females tended to cover longer distances than males (est. = 0.0036, Std. error = 0.0018, t value = 2.028, p = 0.044*; Fig. 2A; Table S4 in Supplementary material).

The natural logarithmic inverse cumulative proportion of moving butterflies decreased with increasing distances and was different for each sex (NEF function: Sex * Distance: t value = −4.982, p = 0.0003***; Fig. 2b; Table S5 in Supplementary material).

Fitting NEF to mobility data resulted in the following equations: Pm = 0.923 e^(–0.0134 D) for males and Pf = 0.6463e^(–0.0079 D) for females (where P is the probability of moving the distance D). The estimated distances ($D' = 1/k$) between consecutive captures were 75 m and 127 m, respectively, for males and females. The estimated probabilities (% of individuals) of long-distance movements were 0.12 % (males) and 1.3 % (females) for 500 m, 0.0002 % (males) and 0.03 % (females) for 1 km, and approximately 0 for both sexes for 2 km (2.3 e–12 % males and 9.9 e–8 % females).

3.3. Sequence polymorphism in genes

Our final data set consisted of 43 sequences of 661 bp for the COI gene and 52 sequences of 349 bp for the nuclear *wingless* gene across the sampling sites in Susa and surrounding Valleys (Appendix Table S1 in Supplementary material). We identified four different COI haplotype characterised by 3 variable nucleotide sites, of which three are new for the species. COI_2 was identified in an individual from LR, COI_3 in two individuals from FF and COI_4 in four individuals from EX and MB (Figure S3a in Supplementary material). COI_1 was the most common haplotype detected in our samples (36 individuals), and it matched with two sequences already published in GenBank (www.ncbi.nlm.nih.gov/genbank, Zinetti et al., 2013, accession number KC119724; Dapporto et al., 2019, accession number MN139729) from North Italy. The haplogroup including all the samples analysed in this study and some other samples from Piedmont is separated from the other European haplogroups (Fig. S3a in Supplementary material).

A 349 bp portion of the *wg* marker was amplified in a subsample of 53 individuals from High (n = 24), Middle (n = 24) and Low (n = 5) Susa Valley. The five identified haplotypes were characterised by 4 variable nucleotide sites; nucleotide diversity (π) was 0.0042. The most represented haplotypes were WG1, WG2 and WG3 (Figure S2b in Supplementary material). These haplotypes were shared among all three areas, while WG4 occurred only in individuals from Middle Susa Valley, and WG5 occurred only in two individuals

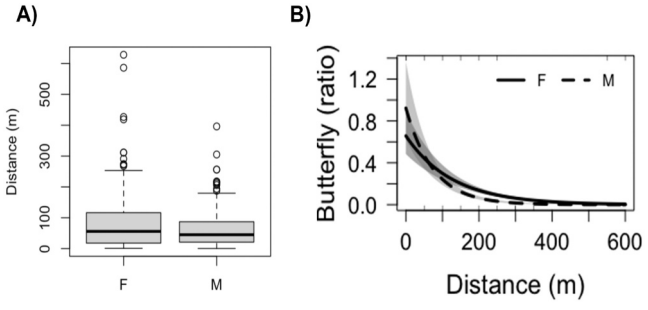

Fig. 2. A) Boxplot of distances covered by females and males of *Zerynthia polyxena*. B) Proportion of female (solid line) and male (dashed line) butterflies captured at distance D.

from High Susa Valley (Fig. S3b in Supplementary material).

Sequences of COI and *wingless* markers were deposited on GenBank (COI accession numbers: PP956960 - PP957002; *wingless* accession numbers: PP964917 - PP964981). Genetic *p-distances* among sampling sites for both markers were low (*p-distances* <0.01, not shown).

3.4. Genetic diversity and subpopulation differentiation

A total of 140 individuals were genotyped, of which a few individuals (n = 4) were represented by a low number of reads; these individuals were removed by PLINK filters. In the first step, the SNP calling pipeline recovered 11.211 SNPs in 140 individuals, then after the filtering procedures and Hardy–Weinberg equilibrium tests in PLINK, 1.629 SNPs for 136 individuals were obtained, and subsequent analyses were carried out with that filtered dataset.

Overall indices calculated on the dataset indicated an observed heterozygosity (H_O) of 0.3391 and an expected heterozygosity (H_E) (or subpopulation genetic diversity) of 0.2441. FortFenil (FF) showed lower values of both H_E (0.1462) and H_O (0.1968) while FortFenil-Pramand (FP) showed higher values (H_E= 0.1462, H_O= 0.4341) (Table 2). The inbreeding coefficients (F_{IS}) were negative in all 14 subpopulations (Table 1) indicating the avoidance of inbreeding and varied between −0.154 (FF) and −0.319 (VG), with a significant difference between the sampling sites (P=0.005). Genetic divergence between groups, measured by Fixation index F_{ST}, showed low values indicating poor differentiation among sampling sites. The values ranged from 0 to 0.113 between FF in High Susa Valley and VG Sangone Valley (Table 3), with an overall F_{ST}= 0.03. Overall, the values highlighted more divergence among subpopulations from Low Susa, Sangone and Lanzo Valley Valleys than those from Middle and High Susa Valley. This is consistent with the moderate and significant pattern of isolation by distance (IBD) found across all sampling sites ($R^2 = 0.32$) from High, Middle, Low Susa Valley, Sangone and Lanzo Valleys (Fig. 3). Instead, no isolation by distance (IBD) was observed across the subpopulations of the High and Middle Valley ($R^2 = 0.013$, not shown).

Discriminant Analysis of Principal Components (DAPC), with membership defined by geographical subpopulation, highlighted three main clusters (Fig. 4), the first including all subpopulations from High and Middle Susa Valley, a second cluster including Low Susa Valley's subpopulations and Lanzo Valley, and a third cluster including Sangone Valley. The DAPC using Bayesian information criterion (BIC, Figure S4 in Supplementary material) suggested that individuals can be attributed to four genetic clusters (K = 4); one cluster includes all individuals from subpopulations of Sangone Valley (VG) and Val della Torre (VDT), seven out of eight individuals from Lanzo Valley (VL) and 5 out of 9 individuals from Milanere (ML) (Figure S4 in Supplementary material). The individuals from high and middle Susa Valley subpopulations were distributed among the other three genetic clusters, with a variable proportion and without a clear overlap with sampling sites. Both Bayesian clustering analyses, with and without prior group information, split the individuals sampled in Susa Valley from sampled individuals in Val della Torre, Sangone and Lanzo Valley with few exceptions (Fig. 4).

The same pattern of divergence was also shown by the analyses of directional genetic differentiation and relative migration conducted with divMigrate (Fig. 5), that indicated low connectivity between Susa and the other valleys and high connectivity among the subpopulations.

The genetic estimated effective subpopulation size (N_e), for middle and high Susa Valley subpopulations, was found to be very low: N_e = 7.1 in the Middle (49 ind. sampled) and N_e = 2.9 in High Susa Valley (32 ind. sampled) (Table 4), and even lower in each subpopulation (N_e values range from 1.8 to 9.2). Global estimate of genetic population size, calculated for the Middle and High Susa Valley, reached 88.0 individuals (Pollak Temporal method).

4. Discussion

In this study, as hypothesised, we found that *Zerynthia polyxena* shows small subpopulations characterised by low mobility, with most individuals travelling less than one hundred metres from their original capture site. There were no observations of marked individuals moving more than 700 m, and movements between sites were very rare. The high isolation of the small subpopulations living

Table 3
Pairwise FST values between *Zerynthia polyxena* subpopulations. In bold, significant Fst (P value <0.001). Site abbreviations as given in Table 1.

Sites	CH	EC	EX	FF	FP	GL	LC	LR	MB	ML	SA	VG	VLT
CH													
EC	**0.016**												
EX	**0.008**	**-0.004**											
FF	**0.037**	**0.044**	**0.035**										
FP	**0.032**	**0.009**	**0.010**	**0.086**									
GL	-0.001	**0.019**	**0.007**	**0.036**	**0.026**								
LC	**0.008**	**0.005**	0.000	**0.049**	-0.001	**0.005**							
LR	**0.007**	**0.030**	**0.016**	**0.010**	**0.063**	**0.012**	**0.031**						
MB	**0.010**	0.000	-0.001	**0.054**	0.003	**0.014**	0.000	**0.027**					
ML	**0.036**	**0.025**	**0.021**	**0.048**	**0.050**	**0.038**	**0.040**	**0.037**	**0.027**				
SA	**-0.004**	**0.006**	-0.003	**0.028**	**0.019**	-0.001	-0.001	**0.005**	0.000	**0.030**			
VG	**0.066**	**0.033**	**0.037**	**0.114**	**0.035**	**0.061**	**0.043**	**0.084**	**0.030**	**0.027**	**0.049**		
VLT	**0.061**	**0.030**	**0.033**	**0.105**	**0.044**	**0.060**	**0.043**	**0.077**	**0.030**	**0.014**	**0.050**	**0.012**	
VL	**0.056**	**0.027**	**0.029**	**0.078**	**0.047**	**0.054**	**0.046**	**0.063**	**0.033**	**0.009**	**0.044**	**0.020**	**0.013**

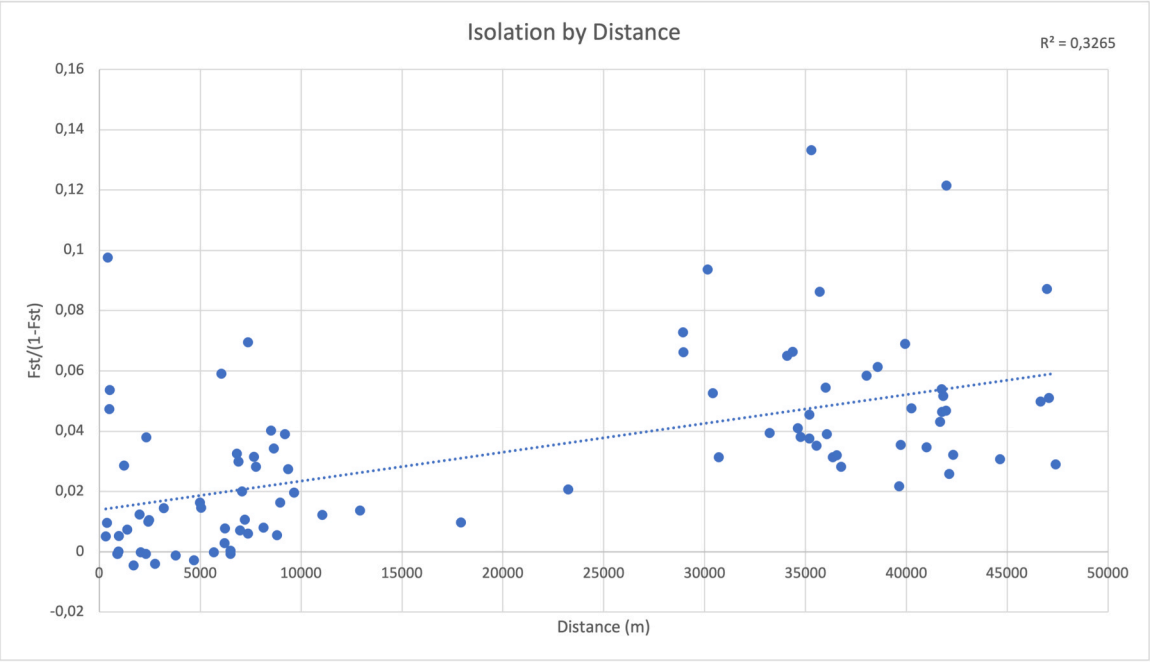

Fig. 3. Isolation by distance, relationship between the linearized FST estimates and geographic distances (m) for the 14 sampling locations of *Zerynthia polyxena* in NW Italy. Genetic variability was geographically structured (p = 0.009).

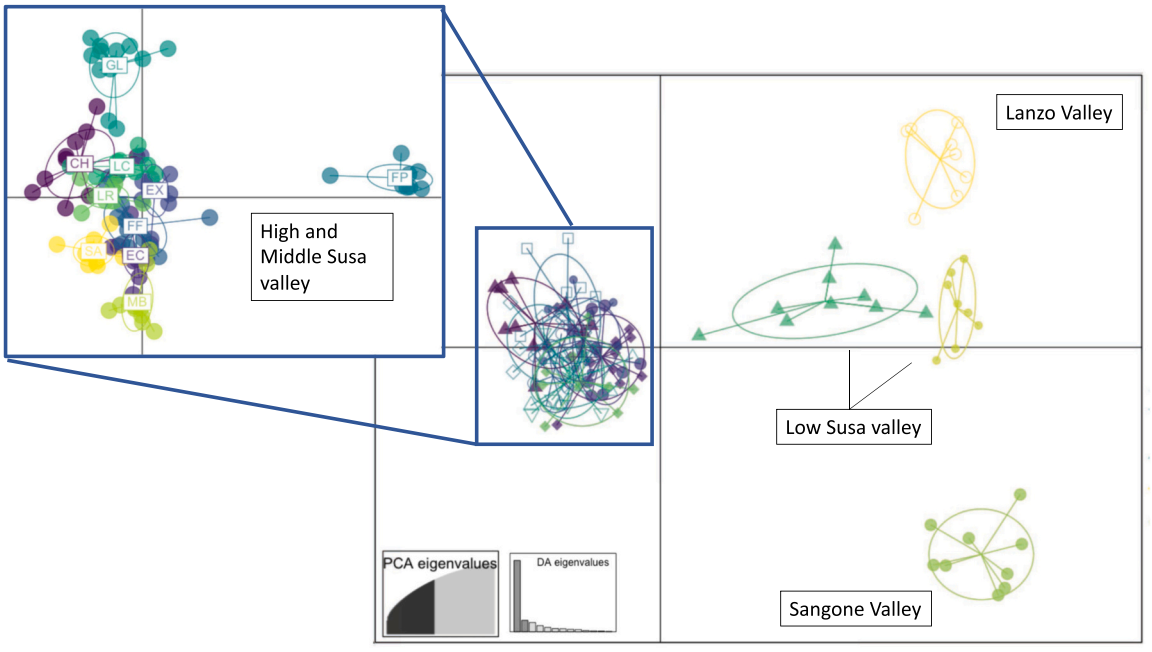

Fig. 4. Discriminant Analysis of Principal Components (DAPC) scatterplot of *Zerynthia polyxena* with group membership defined by site calculated for SNP dataset. The different NW Italian subpopulations are represented by different colours and symbols, each symbol represents different individuals and each subpopulation as inertia ellipses.

in separate sites was not paralleled by strong genetic substructuring and differentiation, nor did we observe a loss of genetic heterozygosity within the separate areas and we did not record genetic bottlenecks as hypothesised. Thus, for the subpopulation threatened by the construction of major infrastructures, it would be feasible to apply conservation measures that might include relocation and the creation of ecological corridors.

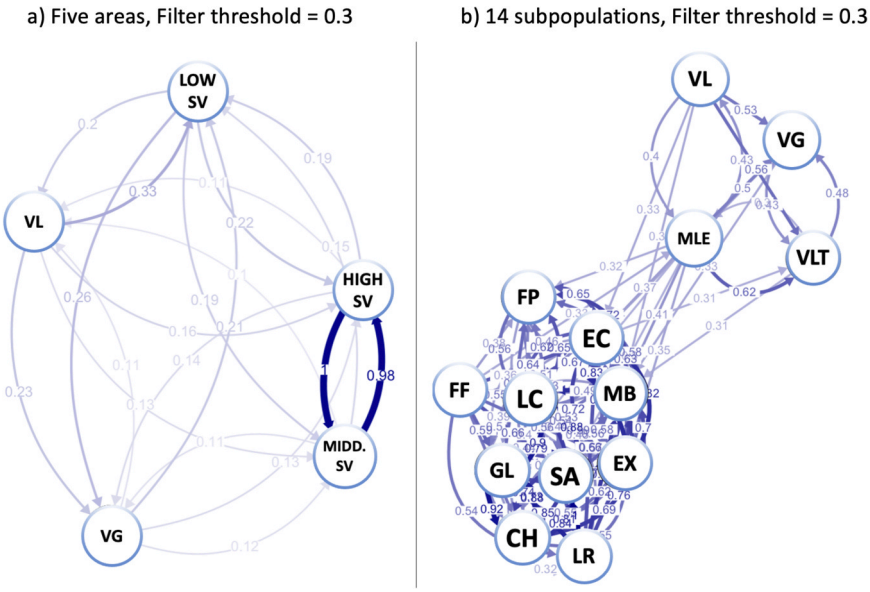

Fig. 5. Directional relative migration network between pairs of *Zerynthia polyxena* subpopulations estimated from genomic data, circles represent sampling sites, and each arrow represents the direction and the relative migration coefficients between the sites derived from the *Nm* statistics. Close subpopulations have more gene flow, the arrow colours indicate relative migration value. **a)** Relative migration rates estimated in 5 areas in NW Italy: High Susa Valley (High SV), Middle Susa Valley (Midd. SV), Low Susa Valley (Low SV), Sangone Valley (VG), Lanzo Valleys (VL). **b)** Relative migration rates, samples grouped in 14 populations (Filter threshold = 0.3). Site abbreviations as given in Table 1.

Table 4
Zerynthia polyxena marked individuals, estimated subpopulation sizes, genotyped individuals and effective population sizes. Site abbreviations as given in Table 1.

	Capture, Mark, Recapture			SNPs		
Area	Sampling sites	N° of marked individuals	Estimated subpopulation size	Genotyped individuals	*Ne*	95 % CIs JackKnife on Loci
High Susa Valley	FF and FP	219	478	17	1.8	0.9 – 5.7
	LC	36	73	5	*infinite*	1.4 - *infinite*
	Total	255	551	22	2.1	1.1 – 6.6
Middle Susa Valley	CH	58	90	10	2.2	1–13
	GL	66	120	12	9.2	2.9 – 40.1
	LR and SA	392	859	17	2.9	1.4 – 12.7
	Total	516	1069	39	3.6	2.2 – 8.5
GLOBAL		771	1620	61	88.0	61.6–142.2

4.1. Estimation of subpopulation sizes

The first adults at the site were recorded on March 18th, and no butterflies were observed after June 12th. The flight season started earlier than previously observed (Čelik, 2012) and lasted more than twice as long as previously recorded for this species (Batáry et al., 2008; Čelik, 2012). These differences are likely related to the extended beneficial weather conditions that ensure suitable temperatures earlier (Roy et al., 2001). Compared to other studies on the same species (Čelik, 2012), the numbers of recaptures were lower (25 % vs. 41 %), with higher recapture rates for males than females. The demographic pattern with a higher male catchability is similar to that observed in other univoltine butterflies (e.g., Baguette and Schtickzelle, 2003; Čelik, 2012; Fric et al., 2010). The lower recapture rate is likely related to the large suitable habitat present in the study area. Indeed, *Z. polyxena* prefers ecotonal areas (Piccini et al., 2022a) and these areas have increased in the Alps due to pastoral and agricultural abandonment. In line with several other studies (Čelik, 2012; Parile et al., 2021; Piccini et al., 2024), the number of marked males was higher than that of females, resulting in an average sex ratio of 1.1. The lifespan (average 4.3 days, min 1.24 days, max 3.81 days) was proved to be similar to other studies on the same species (4.4 days; Čelik, 2012) and shorter than other univoltine butterflies, such as *Aporia crataegi* (Linn. 1758) (about 7 days; Jugovic et al., 2017).

Conversely, despite the high number of breeders estimated by CMR in the subpopulations, the genetically estimated effective subpopulation sizes (Ne) were very low indicating probably genetic drift or small founder populations. Our data are comparable to those found for another strongly sedentary and endangered butterfly, Maculinea alcon (Vanden Broek et al., 2017) and differ noticeably from other butterfly species (Czajkowska et al., 2020; Trense et al., 2021). Effective population size values estimated for our

subpopulations of *Z. polyxena* are lower than the threshold of 50 (with census population size N>500) required to avoid inbreeding depression and the loss of genetic diversity (Ne = 500–5000; N = 5000–50000) (Frankham et al., 2010); the values estimated reach the threshold of 50–500 when we consider the Middle and Low Susa Valley as a metapopulation suggesting that the metapopulation could remain viable at least in the short term. Loss of habitats, as well as anthropogenic and natural barriers, can affect genetic connectivity in animal populations (Trense et al., 2021).

4.2. Mobility and gene flow among subpopulations

The low mobility of *Z. polyxena* (Čelik, 2012; Örvössy et al., 2014; this study) is typical of *Zerynthia* butterflies, being also found in *Z. cassandra* (Vovlas et al., 2014) and *Z. cerisy* (Slancarova et al., 2015). To date, movements just over 600 m were only found once in our study area, and movements over 3 km were reported in one case in *Z. cerisy* and never in *Z. polyxena* in Greece (Slancarova et al., 2015).

In this study, we found differences between female and male dispersal ability in line with the findings of Čelik (2012). Indeed, females fly greater distances and move more frequently between sites than males as they search for suitable oviposition sites. Mating systems and asymmetry in parental investment may lead to sex-biased dispersal, with sexual conflicts which involve female-female competition for feeding or egg-laying areas and male-male competition for sexual partner access (Perrin et al., 2000; Trochet et al., 2013).

In *Z. polyxena* metapopulation, we found a low genetic differentiation between nearby subpopulations indicating that gene flow exists, despite the low mobility of the species and the geographical barriers originated by vegetation separating the clearings where *Aristolochia* host plants grow. Indeed, the low mobility and estimated low dispersal ability, derived from CMR data, is dependent on the sampling effort and patch size (Kuefler et al., 2010). Thus, shrub and wood encroachment might also contribute to making it difficult to sample the complete patch size. Indeed, in our study, gene flow among close subpopulations is still present even if the species seems strongly sedentary, the site sizes are small and the encroachment might be a barrier to movements (similarly to Czajkowska et al., 2020; Trense et al., 2021) and in various vertebrates and invertebrates (Bohonak, 1999). These results suggest that gene flow can occur over distances several times longer than the maximum distance recorded from mark-recapture studies, and the maximum dispersal distance can be higher than that calculated by capture-recapture models (Ugelvig et al., 2012).

Analyses on mitochondrial and nuclear markers identified four haplotypes for COI and five for *wingless* markers. Most of the haplotypes are shared among individuals from the different sampling sites. The results on COI and *wingless* sequences did not show a strong genetic differentiation among subpopulations or Valleys; at the same time, we found a high number of haplotypes, highlighting a high genetic variability in the studied subpopulation.

The heterozygosity values and the not significant F_{is} we found in each subpopulation of our study area suggests that *Z. polyxena* is not currently experiencing inbreeding depression, a factor that increases extinction risk in butterfly populations (Nieminen et al., 2001).

We found a low genetic substructuring at a short distance (1–10 km), and large significant genetic population differentiation only for individuals found in sites located at about 40–50 km distance, suggesting limited gene flow and isolation by distance. The subpopulations clustered in two overall clusters (low vs middle-high valley). This could be explained by the existence, in the past, of a large population that, over time, due to vegetation encroachment and other anthropogenic disturbances, has been divided into small and fragmented subpopulations. For those populations distanced 40–50 km, the isolation has already been recorded from the point of view of genetic and physical distance. However, for those that are closer, we found that the subpopulations are still genetically similar, even though no marked butterflies were recorded passing from one site to another.

5. Conclusions and conservation measures

Our study found the presence of very few genetic differences among the High and Middle Susa Valley subpopulations. Besides, we found a high differentiation between Sangone, Lanzo and Low Susa and Middle-High valley subpopulations (separated by around 30 km), with almost an absence of genetic flow. This suggests the existence of two genetic units, the first grouping all subpopulations in the Middle-High Susa valley, and the second group consisting of those in the Sangone, Lanzo and Low Susa valleys. For the subpopulations in High and Middle Susa Valley, even though no marked butterflies were observed flying from one site to another, the genetic differences are low, meaning that few individuals are still connecting those sites.

A subpopulation in the Middle Susa Valley (CH) is currently under threat due to the construction of the European high-speed railway and the related use of large areas for work sites (Piccini et al., 2022b). Considering the low genetic differentiation, conservation measures may include actions focused to counteracting the two main threats, i.e. subpopulations loss due the railway construction sites and separation of suitable habitats due to encroachment. The loss of subpopulations in the construction sites could be balanced by individual relocation from closer sites to enlarge subpopulation size and/or to colonise new sites (Kuussaari et al., 2015). Possible mitigation actions against the isolation and habitat fragmentation due to encroachment could be the creation of new clearings to shape ecological corridors (Piccini et al., 2023a, 2023b), and the translocation of both *Aristolochia* host plants and *Z. polyxena* butterflies. Moreover, for those sites that present a low density of the species but high presence of the host plant (such as GL site), a relocation of some individuals from the threatened subpopulation (CH) or other close sites might increase the subpopulation size and reduce extinction risk (Bragg et al., 2020; Sgro et al., 2011; Westemeier et al., 1998). The use of genetically closely related subpopulations as reintroduction sources is crucial in restoration programmes for protected and threatened species because genetically distant populations may cause genetic disturbances (Frankham et al., 2004). Alternatively, other possible conservation plans might

include reverting woody vegetation encroachment by supporting traditional pastoral and forest management activities, hence counteracting land abandonment in mountain areas.

An effective long-term conservation plan relies on networks of suitable habitats that are sufficiently close and connected to allow natural dispersal and genetic flow (Junker et al., 2021; Thomas et al., 1992). Indeed, it is essential to identify the current ecological connections, such as ecological corridors, that are expected according to the European Biodiversity Strategy for 2030 to preserve suitable habitats and butterfly metapopulation structure. Indeed, shrub and wood encroachment, that is severe in the NW Alps (Orlandi et al., 2016), might be an ecological barrier that disrupts gene flow, increases genetic heterogeneity among subpopulations and in the future lead to local extinctions (Bartonova et al., 2016). The combined use of insect movement, demographic studies and genetic tools is recommended to assess the flow intensity and direction among subpopulations and estimate landscape features impacting connectivity. In conclusion, considering that ecological insights on habitat selection might not be sufficient to understand metapopulation structures and/or differentiation (Patricelli et al., 2013; Stevens et al., 2010), our results, based on both genetic and ecological tools, constitute a scientific base that could be utilised to design proper conservation measures aimed to minimise the impact on the threatened butterfly subpopulations and connect them.

Author contributions

Irene Piccini designed, performed the research, analysed ecological data, wrote the manuscript, and collected data, helped by Davide Bellone and Viviana Di Pietro, and contributed to the interpretation of the results. Simona Bonelli designed the research, supervised the ecological data analyses and contributed to the interpretation of the results. Marco Cucco provided the interpretation of ecological and genetic results and wrote the paper. Irene Pellegrino performed sample preparation, extraction, sequencing, genetic analyses and contributed to the interpretation of the results and wrote the manuscript. Martina Nasuelli performed sample preparation, extraction, sequencing and genetic analyses. Flavio Mignone developed the GBS protocol and supervised the genomic analyses. All authors contributed to revise the paper.

Data Accessibility and Benefit-Sharing

Unique haplotype data are deposited to NCBI Nucleotide Database (XXXX, sequences ID will be added after the acceptance of the manuscript).

The research collaboration was developed with scientists from laboratories at the University of Turin and University of Eastern Piedmont. The researchers from the University of Turin provided the biological samples and performed the ecological analyses, and the researchers from the University of Eastern Piedmont performed the genetic analyses. All collaborators are included as co-authors and data have been shared with the scientific community and the funding agency.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Haplotype sequences are available on GenBank (https://www.ncbi.nlm.nih.gov), linked to the accession numbers provided in the results section.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.gecco.2024.e03095.

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